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# THE CELL CYCLE PROGRAM OF POLYPEPTIDE LABELING IN *CHLAMYDOMONAS REINHARDTII*

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## ABSTRACT

The cell cycle program of polypeptide labeling in synchronous cultures of wild-type *Chlamydomonas reinhardtii* was analyzed by pulse-labeling cells with  $^{35}\text{SO}_4^-$  or  $[^3\text{H}]\text{arginine}$  at different cell cycle stages. Nearly 100 labeled membrane and soluble polypeptides were resolved and studied using one-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The labeling experiments produced the following results. (a) Total  $^{35}\text{SO}_4^-$  and  $[^3\text{H}]\text{arginine}$  incorporation rates varied independently throughout the cell cycle.  $^{35}\text{SO}_4^-$  incorporation was highest in the mid-light phase, while  $[^3\text{H}]\text{arginine}$  incorporation peaked in the dark phase just before cell division. (b) The relative labeling rate for 20 of 100 polypeptides showed significant fluctuations (3–12-fold) during the cell cycle. The remaining polypeptides were labeled at a rate commensurate with total  $^{35}\text{SO}_4^-$  or  $[^3\text{H}]\text{arginine}$  incorporation. The polypeptides that showed significant fluctuations in relative labeling rates served as markers to identify cell cycle stages. (c) The effects of illumination conditions on the apparent cell cycle stage-specific labeling of polypeptides were tested. Shifting light-grown asynchronous cells to the dark had an immediate and pronounced effect on the pattern of polypeptide labeling, but shifting dark-phase synchronous cells to the light had little effect. The apparent cell cycle variations in the labeling of ribulose 1,5-biphosphate (RUBP)-carboxylase were strongly influenced by illumination effects. (d) Pulse-chase experiments with light-grown asynchronous cells revealed little turnover or inter-conversion of labeled polypeptides within one cell generation, meaning that major polypeptides, whether labeled in a stage-specific manner or not, do not appear transiently in the cell cycle of actively dividing, light-grown cells.

The cell cycle program of labeling was used to analyze effects of a temperature-sensitive cycle blocked (*cb*) mutant. A synchronous culture of ts10001 was shifted to restrictive temperature before its block point to prevent it from dividing. The mutant continued its cell cycle program of polypeptide labeling for over a cell generation, despite its inability to divide.

A dividing cell must duplicate its components during a cell cycle to produce in the next generation daughter cells that are identical to itself. Cellular components can be duplicated in roughly one

of two ways—either by continuous synthesis throughout the cell cycle or by discontinuous synthesis at specific cell cycle stages. During the vegetative cell cycle in *Chlamydomonas reinhardtii*, there is a nearly continuous synthesis and accumulation of some cellular components such as stable RNA (6), while there is a discontinuous synthesis at specific cell cycle stages of other components such as nuclear and chloroplast DNA (7), several elements of the photosynthetic apparatus (1, 2, 5, 20), and certain autoregulated enzymes (16). The discontinuous syntheses of these components occur at predictable times in synchronous cultures and appear, along with various morphological changes, as cell cycle events.

Assessing the progress of a cell through the cell cycle can be a difficult matter since some cell cycle events may be tied to relatively independent “clocks.” Such complexities are most apparent if the growth of a cell has been perturbed by drugs that have stage-specific effects (12) or by conditional mutations that block the progress of a cell through the cell cycle (13). Under such circumstances, certain cell cycle events may continue at regular intervals while others may stop. Ordinarily, to determine cell cycle stage, a single parameter or event is examined, such as DNA content, cell volume, or nuclear morphology. However, when the alignment between cell cycle events is disturbed, single parameter determinations of cell cycle stage obviously become inadequate.

In this paper, we have extended the scope of cell cycle stage determination in *C. reinhardtii* by quantitatively analyzing the major polypeptides that are labeled at specific cell cycle stages. The analysis has been carried out by pulse-labeling cells with  $^{35}\text{SO}_4^-$  or  $[^3\text{H}]\text{arginine}$  at different cell cycle stages and resolving labeled polypeptides by one-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. By such techniques one can follow the labeling patterns of nearly 100 different major polypeptides from both membrane and soluble cell fractions. Some of these polypeptides show considerable fluctuations in rate of labeling during the cell cycle, and their labeling patterns serve to identify particular cell cycle stages. This analysis has been applied to a *cb* mutant which, when shifted to restrictive temperature before its block point, does not divide but nonetheless continues to synthesize protein for a considerable time. This mutant appears to follow a continuing cell cycle pattern of protein synthesis despite its inability to divide.

## MATERIALS AND METHODS

### Cell Growth

*C. reinhardtii* 137C *mt*<sup>+</sup> (a wild-type strain from the collection of W. T. Ebersold) and a *cb* mutant, ts10001 (cloned strain derived by mutagenesis from 137C *mt*<sup>+</sup> and described elsewhere; ref. 13), were used in these experiments. Wild-type or *cb* mutant cells were grown autotrophically at 21–23°C with mixing, and cultures were bubbled with 3%  $\text{CO}_2$  in air. Cells were grown in a modification of high salt medium (21) with a reduced  $\text{SO}_4^{2-}$  concentration ( $17\ \mu\text{M}\ \text{SO}_4^{2-}$ ), referred to as RS-HSM where 0.0020%  $\text{MgSO}_4$  was replaced by 0.0017%  $\text{MgCl}_2$ . (The residual  $\text{SO}_4^{2-}$  in the medium was contributed by components of the minor elements solution.) Asynchronous cell cultures were grown under constant illumination with a light intensity measured at the culture surface of 4,000 lx. Cells were synchronized by subjecting cultures to 12 h light-12 h dark illumination cycles (22). In the temperature shift experiments, *cb* mutant cultures were synchronized at permissive temperature (21°C), then shifted to restrictive temperature (33°C).

Cultures subjected to alternating light-dark cycles were monitored for synchronous growth by periodic sampling and counting of cells with a Celloscope cell counter (Particle Data, Inc., Elmhurst, Ill.) or hemacytometer. An automatic sampling device was used to measure incorporation of radioactive precursors (either  $^{35}\text{SO}_4^-$  or  $[^3\text{H}]\text{arginine}$ ) at various intervals during the cell cycle. The device was actuated by a 9-switch cam timer which controlled a sequence of operations including sample withdrawal, addition of radioactive precursor, mixing and incubation of sample, addition of cold acid, collection of precipitated samples on filters, and washing of filters.

### Labeling of Cells

Cells grown on RS-HSM were labeled with  $^{35}\text{SO}_4^-$  (carrier free stock) or  $[^3\text{H}]\text{arginine}$  (20–40 Ci/mmol) as described in individual experiments. To determine the amount of radioactive label incorporated into protein, cell samples were precipitated in the cold by the addition of TCA to a final concentration of 10%. Acid-precipitated material was collected on Whatman GFC filters (Whatman, Inc., Clifton, N. J.) and washed with 20–40 ml of cold 5% TCA, 20 ml of 95% ethanol, and 90% acetone, until pigments were eluted from the filters.

### Cell Fractionation and Sample

#### Preparation for Electrophoresis

To prepare labeled or unlabeled cell samples for electrophoresis, cells (usually from a 40-ml aliquot) were collected by centrifugation (3,600 g, 3–5 min), resuspended and washed with 10 ml HSM, and recentrifuged. The cell pellet was quick-frozen in a dry ice-ethanol bath and either processed immediately or stored at  $-20^\circ\text{C}$ .

To prepare cell fractions, a modification of the procedure described by Hooper (10) was used. The procedure separates subcellular components into a particulate fraction, composed primarily of chloroplast membranes, and a soluble fraction. Frozen cell pellets were thawed and resuspended in 1 ml of cold TNEMM buffer (50 mM Tris-HCl [pH 7.5], 200 mM NaCl, 1 mM EDTA, 2 mM  $MgCl_2$ , 80 mM 2-mercaptoethanol). The resuspended cells were sonicated for 1 min using a Sonifer cell disruptor (Bronson Instruments, Inc., Stamford, Conn.) with a microtip. The sonicated cells were centrifuged at 40,000 g for 15 min at 2°C. The supernatant fluid (S-40) was decanted and subjected to centrifugation at 100,000 g for 60 min at 2°C. To the green pellet (P-40), suspended in 5 ml TNEMM buffer, cold 40% TCA was added to a final concentration of 10%. The clear, pale yellow supernatant fluid (S-100) resulting from centrifugation at 100,000 g was likewise precipitated by the addition of TCA. The pellet (P-100) was discarded. The S-100 will be referred to, henceforth, as the *soluble* fraction and the P-40 as the *membrane* fraction. For whole cell preparations, thawed cell pellets were suspended in 5 ml TNEMM buffer and precipitated by the addition of cold 40% TCA to a final concentration of 10%.

The acid-precipitated membrane, soluble, or whole cell fractions were collected by centrifugation at 12,000 g for 10 min at 2°C. The precipitates were resuspended, washed twice with 10 ml of 10% TCA, and finally resuspended and washed at room temperature with 90% acetone until pigments were removed. The acetone-washed precipitate was collected by centrifugation. The precipitate was resuspended in 0.1–1.0 ml of 3% SDS, 50 mM Tris-HCl (pH 7.0), 5% 2-mercaptoethanol, and solubilized by heating in a boiling water bath for 3–5 min. Materials in whole cell or membrane fractions not solubilized by this procedure were removed after the heating step by low-speed centrifugation.

### Gel Electrophoresis

Solubilized polypeptides were resolved by SDS-polyacrylamide gel electrophoresis. Electrophoresis was carried out on 1-mm thick slab gels using a discontinuous buffer system similar to that described by Laemmli (17). Stacking gels of 4.5% acrylamide were 1.5 cm long and running gels of 10–12.5% acrylamide were 18 cm long. Ratio of acrylamide to *N,N'*-methylene bisacrylamide in both gels was 37.5:1. Electrophoresis was carried out at room temperature with a current flow regulated at ca. 40 mA. The front, marked by 0.05% bromphenol blue included in the sample, was permitted to migrate ca. 15 cm through the running gel (usually 4–5 h).

Gels were fixed in cold 50% TCA for 30 min, rinsed overnight with 7% acetic acid–20% isopropanol, stained for 1 h with 1% Coomassie Blue, and destained with repeated changes of acetic acid–isopropanol. The resolution of labeled polypeptides on unfixed gels was comparable to that on fixed gels. Gels containing  $^{35}S$ -labeled polypeptides (fixed or unfixed) were dried on filter paper

in a vacuum drying apparatus. Fixed and stained gels containing  $^3H$ -labeled polypeptides were prepared for fluorography by impregnating the gel with 2,5-diphenyloxazole (PPO; ref. 4) and then dried.

### Autoradiography and Autofluorography

Autoradiography of  $^{35}S$ -labeled polypeptides was performed using Kodak RP X-omat x-ray film, and autofluorography with Kodak Royal RP X-omat film (Eastman Kodak Co., Rochester, N. Y.). To insure linear film response of  $^3H$ -radioactivity to film image density, films for autofluorography were presensitized with a filtered light-flash according to Laskey and Mills (19), and exposed at  $-70^\circ C$ .

To compare the relative incorporation of  $^{35}SO_4^{=}$  or [ $^3H$ ]arginine into polypeptides from different samples, all samples were subjected to electrophoresis on the same slab gel. The volume of samples to be compared was adjusted so that each sample contained the same total amount of radioactivity. To insure a linear response of the autoradiograms to  $^{35}S$  and the autofluorograms to  $^3H$ , the films were exposed according to an exposure product, *EP*, calculated as follows:

$$EP = \text{days of exposure} \times \text{cpm (in thousands) in sample.}$$

*EP* was varied depending on the distribution of polypeptides within the gel. For gels in which a few major polypeptides predominated, *EP* was held to 100–200; in gels where the total radioactivity was well distributed, *EP*s from 400–800 were used. Most often several exposures were made varying *EP* in the above ranges. An effort was made to use only bands with an  $A_{540\text{ nm}}$  between 0.1–1.0 as measured by a Gilford 2400 spectrophotometer (Gilford Instrument Laboratories, Inc., Great Neck, N. Y.), a range in which film density was most linear with radioactivity (data not shown).

Autoradiograms and autofluorograms were scanned by a Joyce-Loebl microdensitometer (Joyce, Loebl & Co., Inc., Burlington, Mass.). Peak height in the densitometer tracings was usually used as a measure of radioactivity in a polypeptide band; however, in certain instances (when peaks appeared broad or not symmetrical), areas under the peaks were estimated by cutting and weighing of graph paper. Peak heights were compared between two autoradiograms of the same gel which received different exposures by multiplying the peak height by the appropriate of *EP* for the two exposures.

## RESULTS

### Incorporation of [ $^3H$ ]Arginine and $^{35}SO_4^{=}$ in Asynchronous Cells

[ $^3H$ ]Arginine and  $^{35}SO_4^{=}$  are readily incorporated into protein (acid-precipitable, acetone-insoluble material) in asynchronous autotrophic cultures of *C. reinhardtii*. Enhanced rates of  $^{35}SO_4^{=}$

incorporation were obtained by growing cells in a reduced sulfate medium RS-HSM (containing  $17 \mu\text{M SO}_4^{2-}$ ). Cells grew in RS-HSM at the same rate and to the same extent as in the normally used medium, HSM (containing  $32 \mu\text{M SO}_4^{2-}$ ). Fig. 1 shows that  $^{35}\text{SO}_4^{2-}$  incorporation proceeded linearly after a slight lag (usually imperceptible) at a rate of about  $70 \text{ cpm}/10^6 \text{ cells per min}$  at  $2 \mu\text{Ci/ml } ^{35}\text{SO}_4^{2-}$ . Incorporation of  $^{35}\text{SO}_4^{2-}$  was strikingly light-dependent, for, in the dark, incorporation rapidly dropped to less than 10% of the rate in illuminated cultures. The light requirement for  $^{35}\text{SO}_4^{2-}$  incorporation was of concern since  $^{35}\text{SO}_4^{2-}$  incorporation was to be used in subsequent experiments to examine polypeptide labeling in cells synchronized by alternating light-dark cycles. We found, however, that cells taken from the dark almost immediately acquired the capacity for  $^{35}\text{SO}_4^{2-}$  incorporation when shifted to the light.

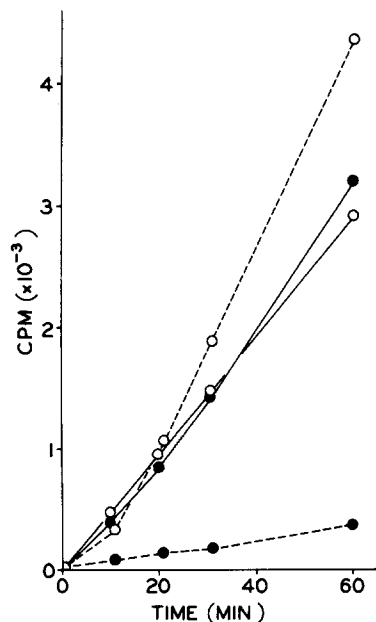


FIGURE 1 Incorporation of  $^{35}\text{SO}_4^{2-}$  and [ $^3\text{H}$ ]arginine in asynchronous cells.  $100 \text{ ml}$  of cells at  $1.0 \times 10^6 \text{ cells/ml}$  were incubated with  $2 \mu\text{Ci/ml } ^{35}\text{SO}_4^{2-}$  in the light ( $\circ\text{---}\circ$ ) and in the dark ( $\bullet\text{---}\bullet$ ) or with  $0.1 \mu\text{Ci/ml } [^3\text{H}]\text{arginine}$  in the light ( $\circ\text{---}\circ$ ) and in the dark ( $\bullet\text{---}\bullet$ ). Cells incubated in the dark were placed in the dark 30 min before the addition of radioactive label. At the time intervals indicated,  $5 \text{ ml}$  of culture was withdrawn, placed on ice, and  $10 \text{ ml } 20\% \text{ TCA}$  was added. The samples were filtered on GFC filters washed with  $5\% \text{ TCA}$ ,  $95\% \text{ ethanol}$ , and  $90\% \text{ acetone}$ . The filters were dried and counted.

Therefore, in all subsequent experiments we labeled cells with  $^{35}\text{SO}_4^{2-}$  in light regardless of their previous exposure to light or dark in the synchronizing illumination cycles. The incorporation of [ $^3\text{H}$ ]arginine into light-grown cells, on the other hand, was relatively insensitive to light effects. During the time-course shown in Fig. 1, [ $^3\text{H}$ ]arginine ( $0.1 \mu\text{Ci/ml}$ ) was incorporated with nearly equal efficiency in the light and dark at a rate of about  $50 \text{ cpm}/10^6 \text{ cells per min}$ .

### Patterns of Polypeptides from Asynchronous Cells

To resolve major whole cell polypeptides, whole cell, soluble, and membrane fractions were analyzed by one-dimensional SDS-polyacrylamide gel electrophoresis. The gel patterns of whole cell preparations stained with Coomassie Blue showed a distribution of polypeptides between  $15\text{--}140\text{K}$  daltons with an abundance of major polypeptides in the ranges of  $22\text{--}33$  and  $45\text{--}55\text{K}$  daltons (Fig. 2). The gel patterns from the fractionated preparations showed that the  $22\text{--}33\text{K}$  dalton bands were largely derived from the membrane fraction and the  $45\text{--}55\text{K}$  dalton bands came largely from the soluble fractions. The membrane polypeptides in the  $22\text{--}33\text{K}$  dalton range correspond to the major chloroplast thylakoid membrane polypeptides called group II polypeptides by Beck and Levine (2), and the major polypeptides at  $55$  and  $15\text{K}$  daltons in the soluble fraction comigrate with the large and small subunits of RUBP-carboxylase.

The general pattern obtained by autoradiography of  $^{35}\text{S}$ -labeled polypeptides (Fig. 5) or autoradiography of  $^3\text{H}$ -labeled polypeptides (Fig. 8) was quite similar to that seen in stained gels. Certain individual polypeptides, which will be pointed out later, were more heavily labeled by  $^{35}\text{SO}_4^{2-}$  than [ $^3\text{H}$ ]arginine or were more radioactively labeled than stained. These differences are thought to relate to the different S or arginine content of the polypeptides. The most pronounced differences between stained and radioactively labeled gels were found in the membrane fraction. More slowly migrating material was found in the  $^{35}\text{S}$ -labeled gels, particularly in the regions above  $120\text{K}$  daltons and near  $50\text{K}$  daltons. Membrane polypeptides near  $50\text{K}$  daltons correspond to group I polypeptides described by Beck and Levine (2).

In the radioactively labeled gels, more than 100

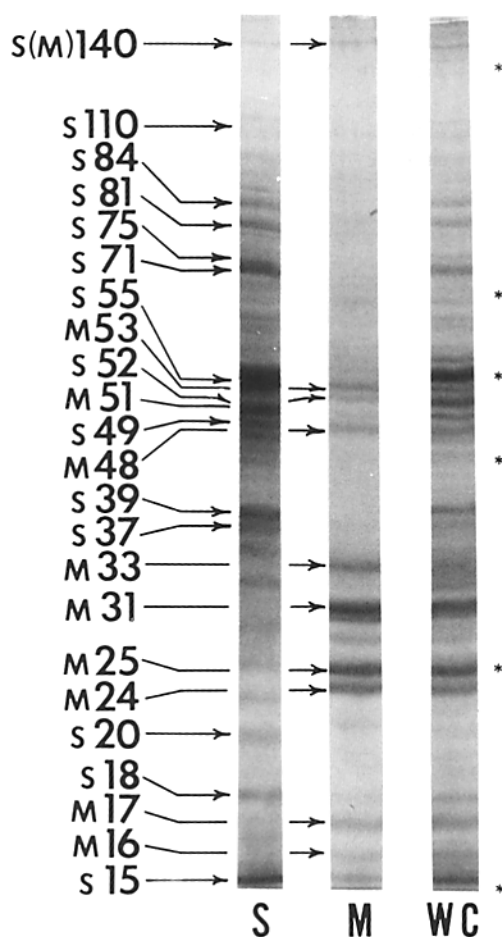


FIGURE 2 Polypeptide pattern from asynchronous cells. Polypeptides from soluble (S), membrane (M), and whole cell (WC) preparations were resolved by electrophoresis on 11% polyacrylamide gels and stained with Coomassie Blue. Polypeptides are designated by the prefixes *s* and *m* and by the molecular mass given in k daltons. Polypeptide molecular weight is related to electrophoretic mobility and based on the migration of standards indicated by asterisks (\*) to the right:  $\beta$ -galactosidase, 130,000; bovine serum albumin, 68,000; RUBP-carboxylase large subunit, 55,000; ovalbumin, 43,000; chymotrypsinogen, 25,700; and ribonuclease A, 14,700.

polypeptides in total can be discerned. Routinely, we have followed about 90 major polypeptide classes seen in the densitometer tracings. In general the resolution of polypeptides, and particularly minor ones, is far greater on radioactively labeled gels than on stained gels. This is because band intensity on labeled gels can be increased by autoradiographic film exposure, while on stained

gels, band intensities relate to the amount of protein in the sample. When more protein is loaded on a gel to intensify weakly staining bands, electrophoretic artifacts and smearing because of overload are found.

#### *Incorporation Rates of $^{35}\text{SO}_4^-$ and $[^3\text{H}]\text{Arginine}$ during the Cell Cycle in Synchronous Cells*

The incorporation rates of  $^{35}\text{SO}_4^-$  and  $[^3\text{H}]\text{arginine}$  during 25 min pulse-labeling periods in the cell cycle are shown in Fig. 3. The incorporation rate of both  $^{35}\text{SO}_4^-$  and  $[^3\text{H}]\text{arginine}$  varied during the cell cycle but in opposite fashion.  $^{35}\text{SO}_4^-$  incorporation quickly reached its highest point midway through the light phase and dropped off rapidly thereafter.  $[^3\text{H}]\text{Arginine}$  incorporation, on the other hand, generally increased during the light phase and peaked in the dark phase before the time of cell separation. The peak in  $^{35}\text{SO}_4^-$  incorporation during the light phase can be attributed both to the stage-specific synthesis of membrane polypeptides and the apparent drop in  $^{35}\text{SO}_4^-$  utilization capacity by dark-phase cells

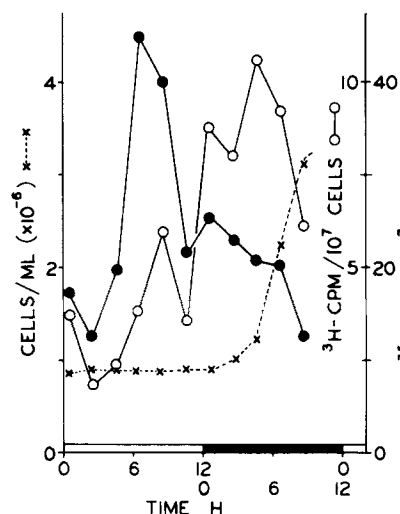


FIGURE 3 Incorporation of  $^{35}\text{SO}_4^-$  and  $[^3\text{H}]\text{arginine}$  during the cell cycle in synchronized cells. 40-ml aliquots of a 1-liter culture synchronized by 12 h light (open bar)-12 h dark (dark bar) were pulse-labeled for 25 min in the light with  $0.2 \mu\text{Ci/ml } ^{35}\text{SO}_4^-$  and  $1.5 \mu\text{Ci/ml } [^3\text{H}]\text{arginine}$ . Cell samples were withdrawn every 2 h, incubated, and processed by the cell sampling device described in Materials and Methods. Midpoint in rise of cell density is designated as the time of cell separation (about D7).

(even though the cells were pulse-labeled in the light). During the light phase,  $^{35}\text{SO}_4^-$  incorporation into membrane polypeptides accounts for nearly 45% of the total incorporation, whereas during the rest of the cycle, only about 20% of the incorporation is found in this fraction.

#### *Patterns of Polypeptides Labeled at Different Cell Cycle Stages*

To determine when various polypeptides are labeled during the cell cycle, cell samples from different stages in a light-dark synchronized culture were pulse-labeled for 25 min *in the light* with  $^{35}\text{SO}_4^-$ . Labeled cells were fractionated into soluble and membrane fractions, and samples containing acid-precipitable polypeptides were prepared for gel electrophoresis. Sample volumes were adjusted so that each sample contained the same amount of radioactivity. In this way, by comparing band intensity or densitometer peak height in various samples, one can determine the change in rate of  $^{35}\text{S}$  labeling of a single polypeptide relative to all others in that cell fraction.

Gel densitometer tracings of membrane and soluble polypeptides obtained from synchronous cells, pulse-labeled at diametrically opposed cell cycle stages (L5 and D5), are shown in Fig. 4. One can easily see in the membrane fraction of mid-light phase cells (L5), large  $^{35}\text{S}$ -labeled peaks, m25, m31, and m33, and associated minor peaks. In mid-dark phase cells (D5) nearing the stage of cell division (about D7), these peaks are highly reduced and much  $^{35}\text{S}$  label in the membrane fraction has been incorporated in a broad, slowly migrating peak at the top of the gel labeled m140. Less pronounced changes in labeling of soluble polypeptides are seen at these two cell cycle stages (Fig. 4); however, the most apparent changes are found in s39, s55, s110, and s140.

A complete set of gels representing  $^{35}\text{SO}_4^-$  pulse labeling of membrane and soluble polypeptides at 2-h intervals throughout the cell cycle is shown in Fig. 5. In this figure the rise and fall in labeling of stage-specific polypeptides can easily be seen, and peak incorporation stages for these polypeptides are indicated by arrows. Changes in the rate of relative labeling of selected polypeptides which show significant fluctuations during the cell cycle are plotted in Fig. 6 (membrane polypeptides) and Fig. 7 (soluble polypeptides). In these figures relative peak heights (or relative labeling) from densitometer tracings have been plotted against cell

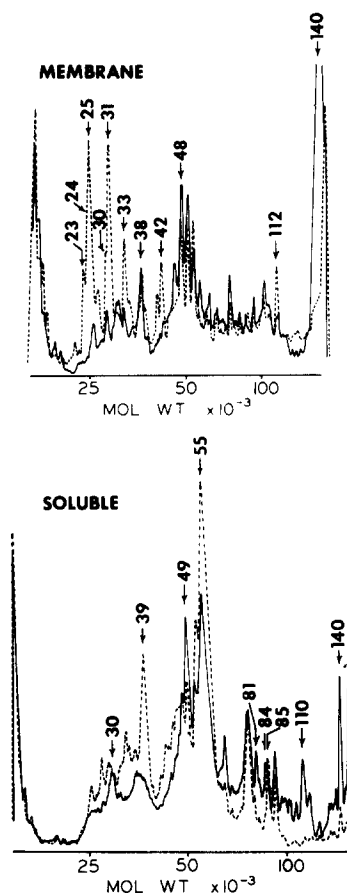
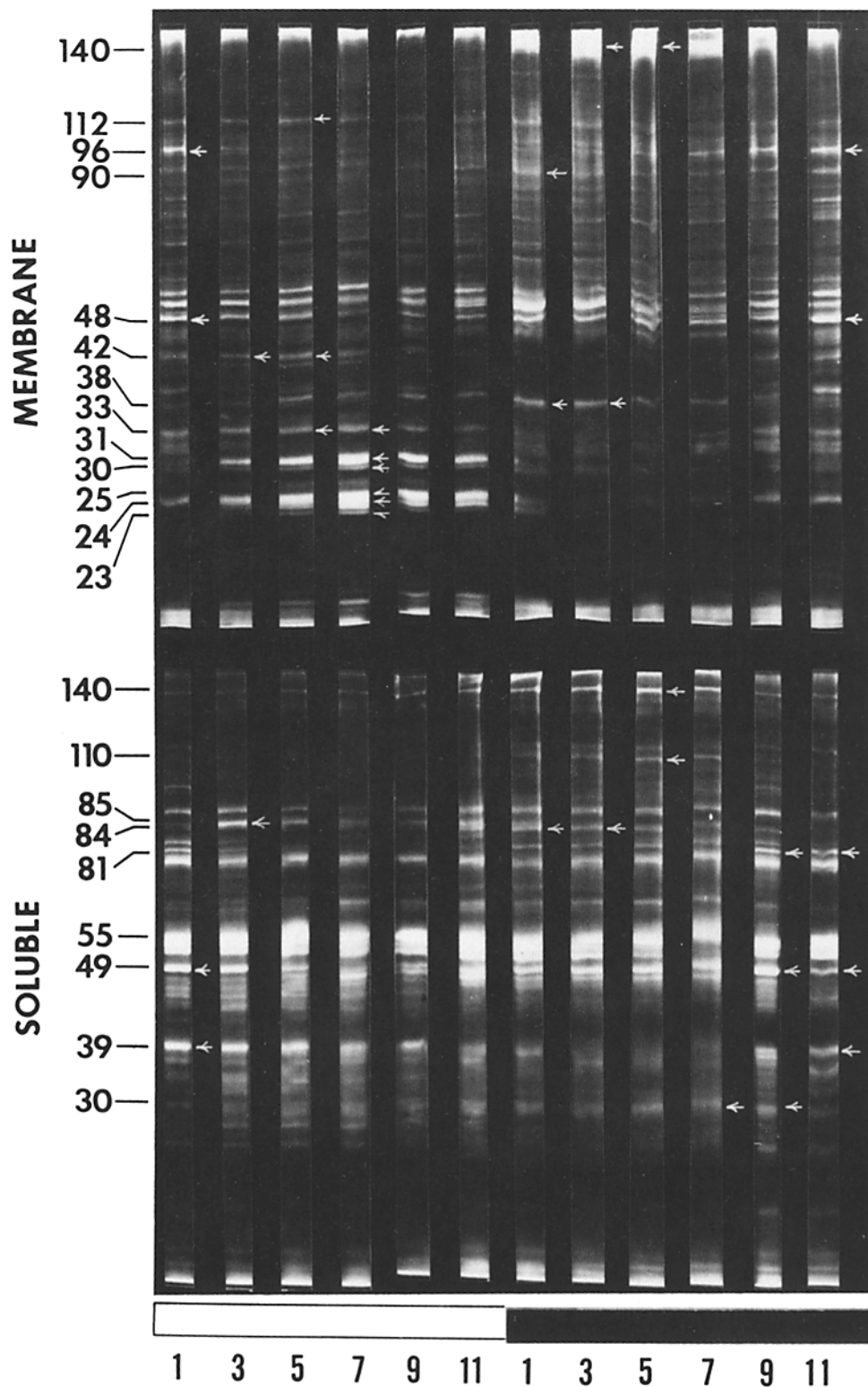


FIGURE 4 Pattern of  $^{35}\text{S}$ -labeled polypeptides from two different cell cycle stages. Shown are densitometer tracings of autoradiograms obtained from 11% polyacrylamide gels which display the pattern of  $^{35}\text{S}$  pulse-labeled membrane and soluble polypeptides from synchronous cells labeled at L5 (-----) and D5 (—). To pulse label cells, 40-ml aliquots of a 1-liter culture (starting L1 at  $8 \times 10^5$  cells/ml) synchronized by 12 h light and 12 h dark were incubated for 25 min *in the light* with  $20 \mu\text{Ci/ml } ^{35}\text{SO}_4^-$ . Samples were processed for electrophoresis, and sample volumes adjusted so that the same amount of radioactivity was applied to each gel channel. Scale of molecular weights on abscissa is related to electrophoretic mobility based on migration of standards as in Fig. 2.

cycle stage. Relative peak height values have been normalized by setting the lowest cell cycle stage value to one. Normalization, thereby, permits one to read easily out the "fold" change during the cell cycle in the labeling rate of any polypeptide compared to all others in that cell fraction. Many of the selected polypeptides showed only about a



**FIGURE 5** Patterns of  $^{35}\text{S}$ -labeled polypeptides during cell cycle in synchronous cells. Shown are autoradiograms of 11% polyacrylamide gels which display membrane and soluble polypeptides pulse-labeled with  $^{35}\text{SO}_4^{2-}$  at 2-h intervals throughout the cell cycle. Conditions for labeling are described in Fig. 4. Samples were processed for electrophoresis, and sample volumes containing the same amount of radioactivity were applied to the gels. Arrows represent stages of maximum relative labeling for polypeptides that show significant labeling fluctuations during the cell cycle. Midpoint of cell separation is at about D7.



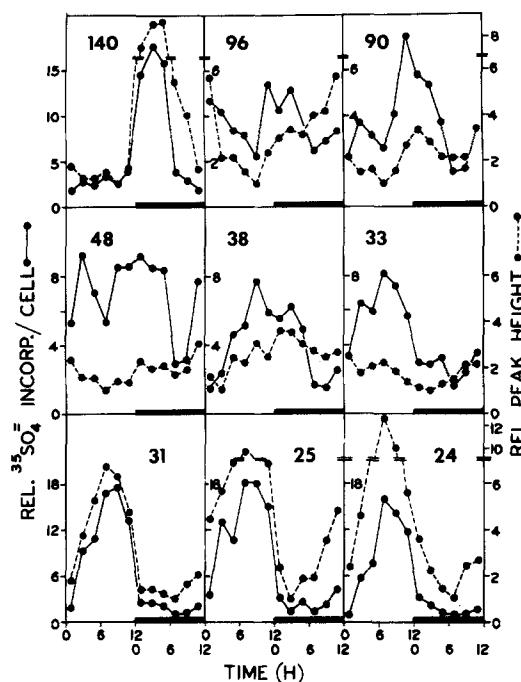


FIGURE 6 Rates of labeling selected *membrane* polypeptides with  $^{35}\text{SO}_4^-$  during the cell cycle. Peak heights from densitometer tracings similar to those in Fig. 4 were plotted against time in cell cycle. Peak height values were normalized such that the lowest value during the cell cycle equals one. Relative peak height at any cell cycle stage expresses a labeling rate of the selected polypeptide with respect to all others in that fraction, since gels are loaded with samples that contain the same amount of radioactivity. Relative  $^{35}\text{SO}_4^-$  incorporation/cell for a selected polypeptide was calculated by multiplying the peak height times the relative amount of total  $^{35}\text{SO}_4^-$  incorporated in the membrane fraction at the corresponding cell cycle stage. Relative  $^{35}\text{SO}_4^-$  incorporation patterns express changes in actual rate of labeling of polypeptides during the cell cycle.

threefold change in relative peak height during the cell cycle, while some of the others, such as m140, m31, m25, m24, s140, s110, and s81, showed an 8–12-fold change.

To determine change in the actual rate of  $^{35}\text{S}$  incorporation into any polypeptide during the cell cycle, peak height was multiplied by the amount of  $^{35}\text{S}$  incorporated into that fraction. The rate of  $^{35}\text{S}$  incorporation into any given membrane polypeptide was largely dependent on the total incorporation into the membrane fraction which varied during the cell cycle as discussed previously. Because of this correction in actual labeling rates (indicated as  $\text{rel. } ^{35}\text{SO}_4^- \text{ incorp./cell}$ ), the patterns in peak

height and  $^{35}\text{S}$  incorporation for a given polypeptide might not coincide in the cell cycle as was found in m96, m48, m38, and m33. The differences in patterns arose when the rate of relative labeling of a polypeptide was high at a stage when the overall  $^{35}\text{SO}_4^-$  incorporation was low. Membrane polypeptides that showed high relative labeling rates during the mid-light phase, when overall  $^{35}\text{SO}_4^-$  incorporation rates were high, exhibited even greater changes in actual  $^{35}\text{SO}_4^-$  incorporation rates. For example, the peak height (or relative labeling rate) varied about 10-fold for m25 during the cell cycle; however, the actual rate of  $^{35}\text{S}$  incorporation in m25 varied more than 18-fold.

A similar set of experiments was performed to study  $[^3\text{H}]$ arginine labeling of polypeptides during the cell cycle.  $[^3\text{H}]$ Arginine offered the advantage that it was as efficiently incorporated in the light or dark during a pulse-labeling period; but the disadvantage that, at reasonable costs, polypeptides could not be labeled to the same specific activity as with  $^{35}\text{SO}_4^-$ . For cell cycle studies, cells were not fractionated since incorporated  $[^3\text{H}]$ arginine was fairly uniformly apportioned between membrane and soluble fractions in both light- and dark-phase synchronized cells.

A complete set of gels representing  $[^3\text{H}]$ -arginine labeling of whole cell polypeptides at 3-h intervals throughout the cell cycle is shown in Fig. 8. In this figure, the stage-specific labeling of sev-

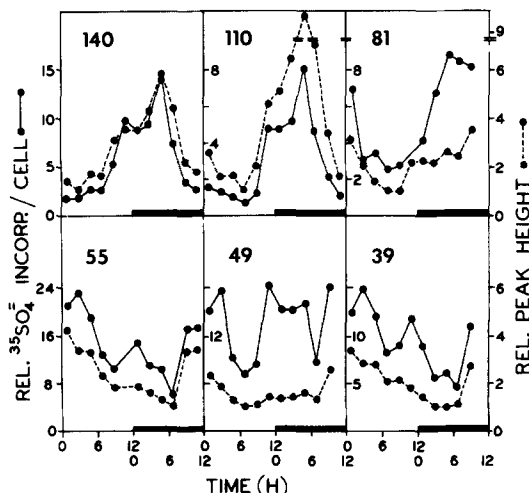


FIGURE 7 Rates of labeling selected *soluble* polypeptides with  $^{35}\text{SO}_4^-$  during the cell cycle. Relative peak height and  $^{35}\text{SO}_4^-$  incorporation/cell values obtained as described in Fig. 6.

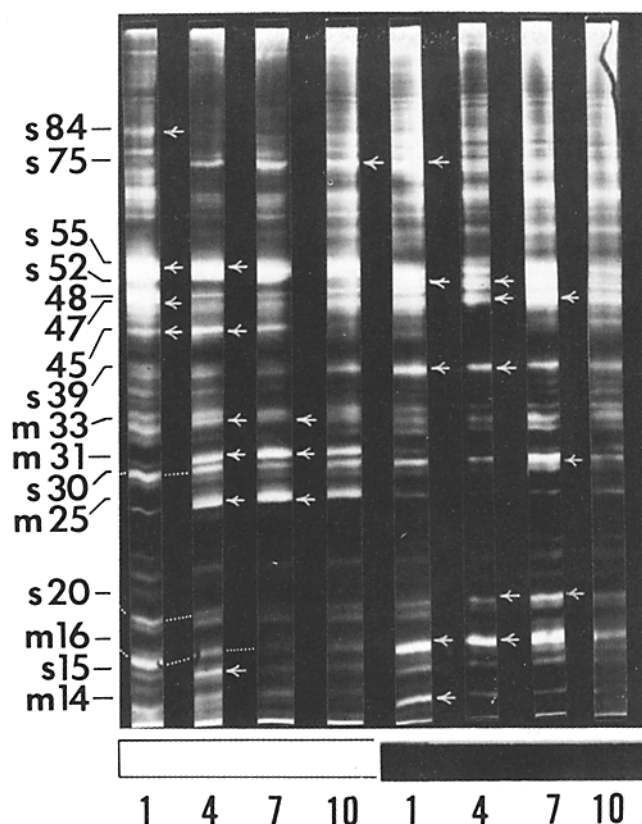


FIGURE 8 Patterns of [ $^3\text{H}$ ]arginine-labeled polypeptides during the cell cycle in synchronous cells. Shown are autoradiograms of 10% polyacrylamide gels which display whole cell polypeptides pulse-labeled with [ $^3\text{H}$ ]arginine at 3-h intervals throughout the cell cycle. To label cells, 50-ml aliquots of a 1-liter light-dark synchronized culture were incubated for 25 min with 2  $\mu\text{Ci/ml}$  of [ $^3\text{H}$ ]arginine *under ongoing illumination conditions*. Samples were processed for electrophoresis and sample volumes adjusted so that the same amount of radioactivity was applied to each gel channel. Arrows represent stages of maximum relative labeling for polypeptides that show significant labeling fluctuations during the cell cycle. Categorization of polypeptide as membrane or soluble was from a cell fractionation experiment such as that shown in Fig. 11. Cell separation occurred at about D7.

eral polypeptides can be seen, and peak stages of labeling are indicated by arrows. Among the polypeptides that show major labeling changes are s15, m20, m25, s30, m31, m33, 47, s52, and s55. As with the previous set of polypeptide gels labeled with  $^{35}\text{SO}_4^-$  at different cell cycle stages, these gels demonstrated that nearly every cell cycle stage was marked by the appearance of one or more polypeptides that were highly labeled at that time. Changes in the relative rate of [ $^3\text{H}$ ]arginine incorporation of six selected polypeptides that exhibited stage-specific labeling are shown in Fig. 9. As before, relative peak height (or relative labeling rate) obtained from densitometer tracings were plotted against time in the cell cycle. (Rela-

tive peak height values were normalized again so that the lowest value was one.) For the polypeptides shown here, the variation in relative peak height ranged from three- to fivefold with the greatest peak height changes in m16, s52, and s55.

Changes in the actual rate of [ $^3\text{H}$ ]arginine incorporation into any polypeptide were also largely dependent on the overall rate of [ $^3\text{H}$ ]arginine incorporation into the cells during the cell cycle. (The relative rate of [ $^3\text{H}$ ]arginine incorporation into any polypeptide is the product of the peak height times the total incorporation.) For example, m25, which showed a high relative labeling rate in the light phase, actually incorporated

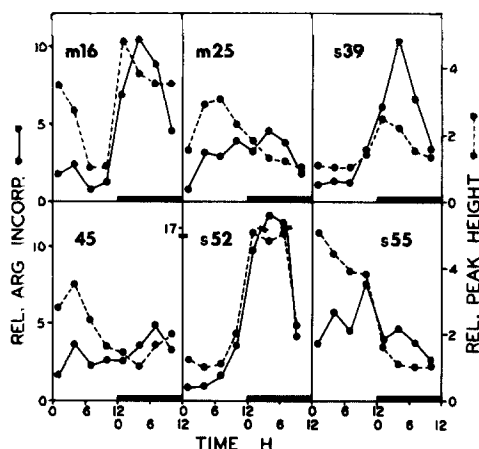


FIGURE 9 Rates of labeling selected polypeptides with  $[^3\text{H}]$ arginine during the cell cycle. Relative peak height and  $[^3\text{H}]$ arginine incorporation/cell values were obtained as described in Fig. 6 for  $^{35}\text{SO}_4^-$  labeling.

$[^3\text{H}]$ arginine at a slightly greater rate in the dark phase (Fig. 9). For polypeptides such as m16 and s52 in which the relative peak height changes follow the pattern of total  $[^3\text{H}]$ arginine incorporation, the actual changes in  $[^3\text{H}]$ arginine incorporation were much greater. The actual rate of  $[^3\text{H}]$ arginine incorporation into s52 was 25 times greater at D5 than L5, while there was only a fivefold change in relative peak height between those times. Likewise, for any polypeptide that showed a constant peak height over the cell cycle, the actual rate of  $[^3\text{H}]$ arginine incorporation into that polypeptide followed the total incorporation pattern shown in Fig. 3.

#### Light and Dark Effects

When light-grown asynchronous cells were pulse-labeled with  $[^3\text{H}]$ arginine in either the light or dark, dramatic differences in the pattern of labeling of major polypeptides were found (Fig. 10). The most prominent pattern differences could be observed in the soluble fraction. Virtually none of the major polypeptides that incorporated  $[^3\text{H}]$ arginine in the light did so in the dark and vice versa. In Fig. 10, it can be seen that s15, s20, s30, s39, s45, s46, s52, s54, s55, s61, and s71 were all extensively affected by an immediate change in illumination conditions. Of the membrane polypeptides, the major cluster of lower molecular weight polypeptides, m33, m31, and m25 apparently required light for efficient  $[^3\text{H}]$ arginine incorporation.

The capacity for pulse-labeling several polypeptides either decayed or emerged when asynchronous cells were placed in the dark for a period of time. In Fig. 11, light-grown asynchronous cells were placed in the dark and pulse-labeled at 0, 4, and 8 h thereafter with  $[^3\text{H}]$ arginine in the light or in the dark. The arrows in the figure point out those polypeptides in which the capacity for

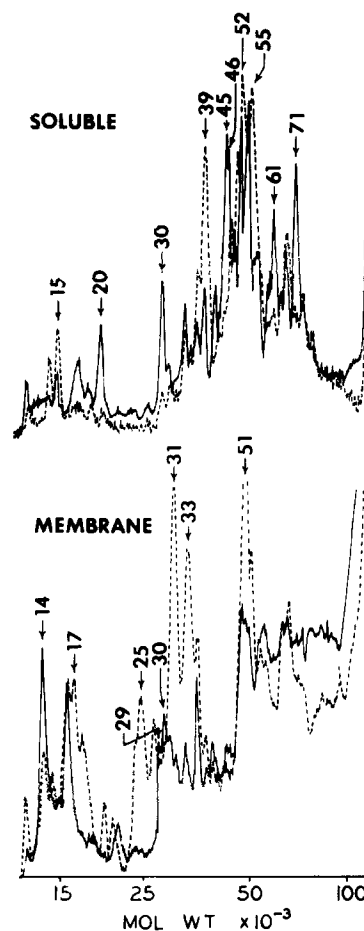


FIGURE 10 Immediate effects of light shift on the labeling pattern of soluble and membrane polypeptides in light-grown asynchronous cells. 40-ml Aliquots from a 1-liter culture of asynchronous cells (at  $1 \times 10^6$  cells/ml) were pulse-labeled for 25 min with  $5 \mu\text{Ci/ml}$   $[^3\text{H}]$ arginine in the light (—) or immediately after shifting to the dark (---). Shown are densitometer tracings of autoradiographs from 10% polyacrylamide gels. Selected polypeptides that exhibit significant labeling changes in the light and dark are indicated. Scale of molecular weights on abscissa is related to electrophoretic mobility based on the migration of standards as in Fig. 2.

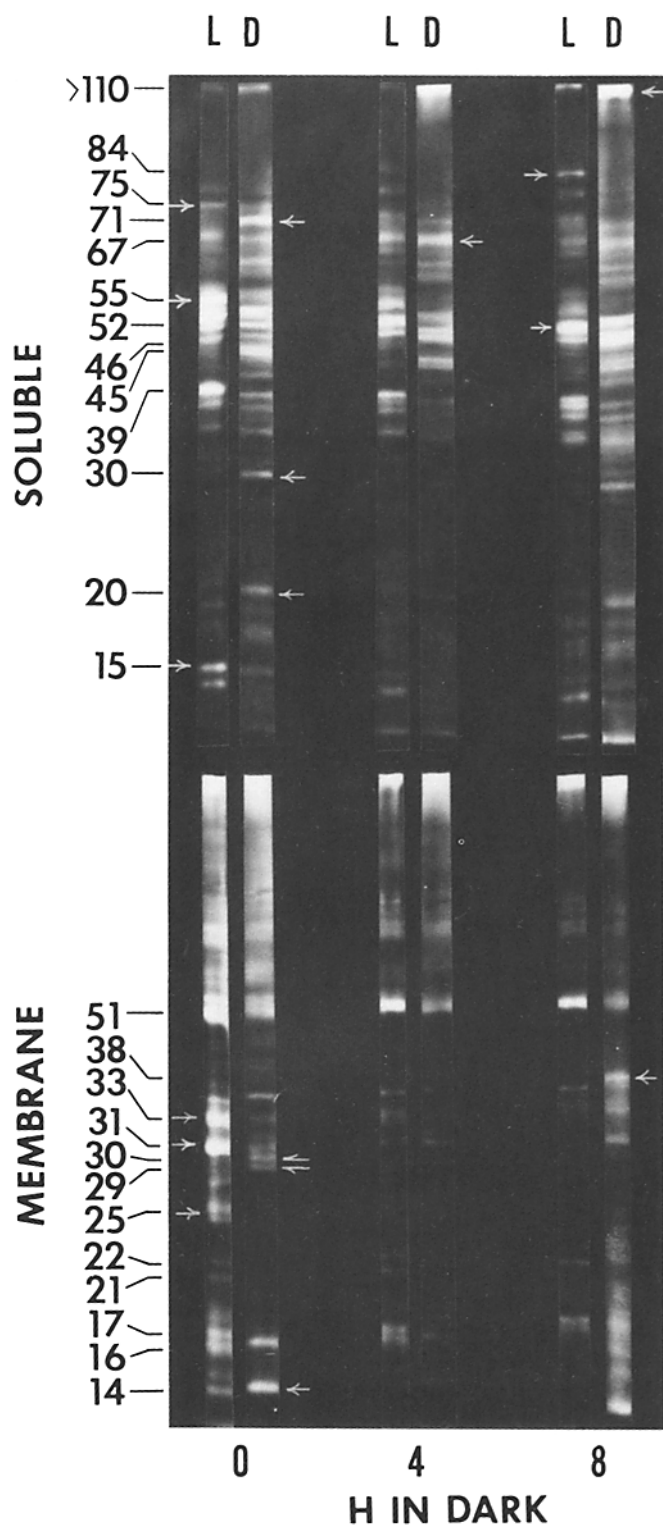


FIGURE 11 Immediate and longer term effects of light-shift on asynchronous cells. Light-grown asynchronous cells were pulse-labeled with [ $^3\text{H}$ ]arginine in the light (*L*) and dark (*D*) according to conditions described in Fig. 10. Light-grown asynchronous cells were also shifted to the dark and at various times thereafter (as indicated) pulse-labeled in either the light or dark. Shown are autoradiograms of membrane and soluble polypeptides resolved on 10% polyacrylamide gels. Polypeptides that show significant increases or decreases in relative labeling rate during the period in the dark are indicated by arrows.

[ $^3\text{H}$ ]arginine labeling in the light or the dark changed with time in the dark. Thus, in the case of s55 or m31, which required light for labeling, when cells were placed in the dark for varying periods of time and pulse-labeled at times thereafter *in the light*, the capacity for labeling these polypeptides decayed with time in the dark. On the other hand, s71, s30, s20, m30, m29, and m14 were labeled almost exclusively in the dark, but the capacity for labeling these polypeptides *in the dark* decayed in the dark. The relative capacity to label several polypeptides increased with time in the dark. This was true for s84 and s52, which were labeled in the light, and s67 and m38, which were labeled in the dark. Thus, the effect of dark on light-grown asynchronous or mid-light phase synchronous cells (data not shown here) was profound, both in terms of its immediate effect on the labeling pattern of polypeptides in the light and dark and in its longer term effects on the decay or emergence of capacity for labeling certain polypeptides in the light.

Light had fewer immediate effects on dark-phase synchronous cells. In fact, in dark-phase cells only two major polypeptides immediately responded to the change in illumination conditions (Fig. 12). Those two polypeptides were s15 and s55 (the large and small subunits of RUBP-carboxylase) which were clearly labeled more extensively in dark-phase cells in the light than in the dark.

#### *Turnover of Polypeptides during the Cell Cycle*

To determine the extent of turnover of labeled polypeptides in cycling cells, pulse-chase labeling experiments with light-grown asynchronous cells were performed. Asynchronous cells were pulse-labeled with  $^{35}\text{SO}_4^-$  in RS-HSM for 25 min in the light. Cells were collected by centrifugation at the end of the labeling period, washed twice with HSM, and resuspended in RS-HSM. Incubation of the resuspended cells was continued in the light and, at various times during the chase period, samples were taken to determine the retention of acid precipitable  $^{35}\text{S}$  radioactivity and the electrophoretic gel pattern of labeled polypeptides.

The chase procedure effectively blocked further  $^{35}\text{SO}_4^-$  incorporation, and it was surprising to find that there was no detectable loss in acid precipitable  $^{35}\text{S}$  incorporation (per aliquot of cell culture) during the 24-h chase period in the light (not

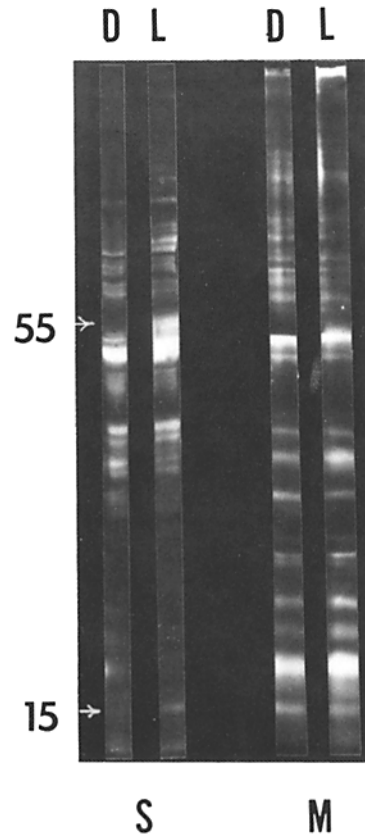


FIGURE 12 Immediate effect of light shift on the labeling of soluble (*S*) and membrane (*M*) polypeptides in dark-phase (D7) synchronous cells. Dark-phase (D7) cells (at  $9 \times 10^6$  cells/ml) were pulse-labeled with 5  $\mu\text{Ci}$ /ml [ $^3\text{H}$ ]arginine in the light (*L*) or dark (*D*). Shown are autoradiograms obtained from 11% polyacrylamide gels. Two polypeptides are indicated which show significant changes in relative labeling rate when shifted to light.

shown here). As well, the gel patterns of labeled polypeptides obtained from cells during the chase did not change significantly (Fig. 13). In the densitometer tracings from 9- and 24-h gels, some peak broadening can be seen, but this broadening probably resulted from the increased load of protein on the gels. Samples contained the same amount of radioactivity, and increased loading of protein at later chase periods resulted from an increasing cell mass per volume of culture while the amount of radioactivity remained constant. One could mimic the peak-broadening effect with the 0-h sample by mixing appropriate quantities of unlabeled cells with labeled cells before processing. Despite the

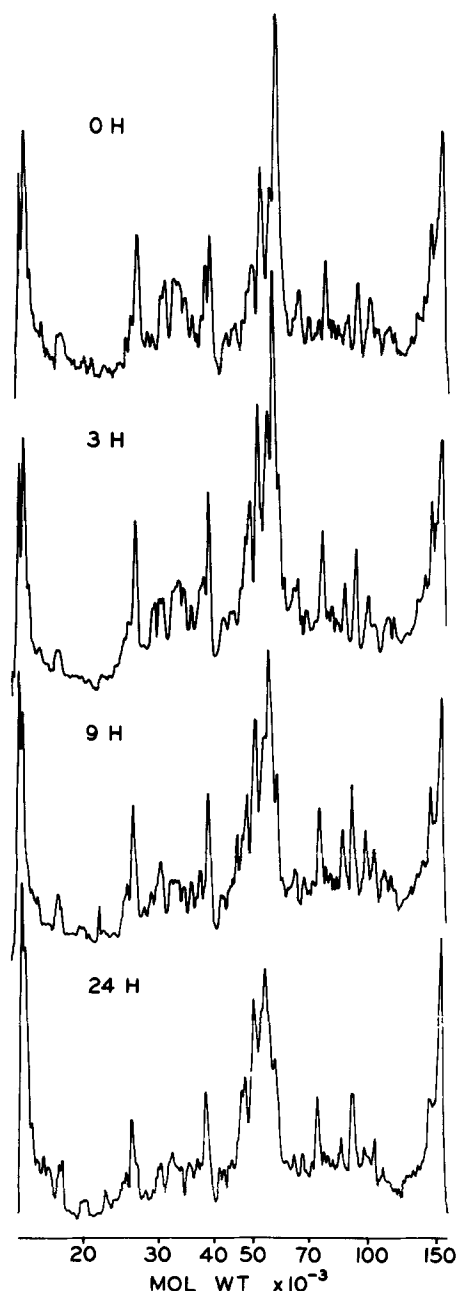


FIGURE 13  $^{35}\text{SO}_4$  pulse-chase experiment with asynchronous cells. A 40-ml aliquot from an asynchronous light-grown culture (at  $2 \times 10^8$  cells/ml) in RS-HSM was incubated with  $20 \mu\text{Ci/ml}$   $^{35}\text{SO}_4$  for 25 min. Labeled cells were rapidly harvested by centrifugation at room temperature, washed twice with the same volume of HSM, and finally resuspended in 200 ml of RS-HSM. The culture was further incubated in the light and, at times indicated, 40 ml of cells was harvested, prepared

peak-broadening effects, the area under peaks of the major polypeptide classes remained nearly the same during the chase.

Therefore, the pulse-chase experiments have revealed that under constant illumination conditions, there is little turnover of acid precipitable  $^{35}\text{S}$  radioactivity within the period of a single cell cycle (ca. 20 h in asynchronous and 24 h in synchronous cultures). Furthermore, there is little interconversion of one polypeptide class to another during that period of time. The absence of appreciable turnover of  $^{35}\text{S}$  polypeptides has also been revealed in several experiments (not shown here) with asynchronous cells comparing pulse-labeled with long-term labeled (24 h) polypeptides. The banding patterns on gels of short- and long-term labeled polypeptides are nearly identical.

It was not investigated in these experiments whether  $^{35}\text{S}$ -labeled, acid precipitable polypeptides were retained with the cells or shed into the medium during the chase. Recent experiments by Lang and Chrispeels (18) have shown that labeled cell wall polypeptides are released at terminal cell cycle stages when the mother cell wall is cast off into the medium.

#### Pattern of Polypeptide Labeling in a *cb* Mutant

In these experiments, the effects of a temperature-sensitive *cb* mutant on the cell cycle pattern of polypeptide labeling with  $[^3\text{H}]$ arginine were examined. The mutant chosen for study, ts10001, had a block point at 0.3 in the cell cycle and continued to incorporate  $[^3\text{H}]$ arginine for more than 24 h (a complete cell generation) after shift to restrictive temperature. (The block point is the cell cycle stage in a *cb* mutant in which cell division becomes insensitive to restrictive temperature conditions; ref. 12.)

The scheme for this experiment, illustrated in Fig. 14, was to shift a synchronized culture of ts10001 from permissive ( $21^\circ\text{C}$ ) to restrictive ( $33^\circ\text{C}$ ) temperature a few hours before the block

for electrophoresis, and the entire sample loaded on gels. By 24 h of chase the incubated cells had completed one cell generation. Shown here are densitometer tracings of autoradiograms from 11% polyacrylamide gels. Scale of molecular weights on abscissa is related to electrophoretic mobility based on migration of standards as in Fig. 2.

point. The culture would then be sampled at various times after temperature shift (indicated L1, L6, D4, etc.) to determine the pattern of [ $^3\text{H}$ ]arginine polypeptides. Under restrictive temperature conditions, the subsequent cell separation in the mutant cells was completely blocked, and the question was whether the cells would continue in their cell cycle program of polypeptide labeling. Wild-type cells were relatively unaffected by the temperature shift with regard to their capacity for cell division and in the pattern of polypeptides labeled (data not shown). The rate of progress through the cell cycle was remarkably similar for the wild-type cells at both temperatures, which was an important criterion for initially establishing the restrictive and permissive temperature conditions.

The pattern of polypeptide labeling in ts10001 cells at permissive and restrictive temperature is shown in Fig. 15. The remarkable feature is the similarity of gel patterns obtained at permissive and restrictive temperature. This pattern is seen to continue relatively intact even up to the last sampling at L6 which was over 30 h or  $1\frac{1}{4}$  cell cycles after the temperature shift. The major polypeptides that gave evidence of a common pattern are indicated by arrows in Fig. 15. One major polypeptide, however, was not labeled at restrictive temperature after division and that was s84 which appears to be missing at the second L1 sampling.

## DISCUSSION

### *Polypeptide Labeling Program in Wild-Type Cells*

Light-dark synchronized cultures of wild-type *C. reinhardtii* display a changing pattern of specific polypeptide labeling which recurs during each cell cycle. This complex pattern documents the changing expression of the genome during the cell cycle and expands the set of indicators with which the progress of a cell through the cell cycle can be demonstrated.

The analysis of the stage-specific labeling of polypeptides in wild-type cells raises some interesting points about the program of polypeptide synthesis in synchronized cultures. First, not all major polypeptides show significant changes in their relative rate (rate relative to that of all other polypeptides in the cell or cell fraction) of labeling during the cell cycle. In fact, only about 30 of nearly 100 major cellular polypeptides show sig-

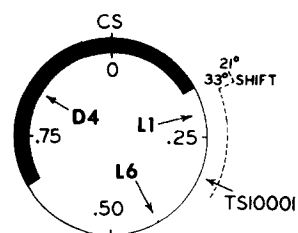


FIGURE 14 Temperature shift experiment with synchronous culture of *cb* mutant, ts10001. Cells are synchronized at permissive temperature ( $21^{\circ}\text{C}$ ) by 12 h light-12 h dark (dark bar), and the cell cycle is represented as a clock that progresses from zero, the time of cell separation (CS), to one. The block point for ts10001 at 0.3 is indicated by the arrow. Culture is shifted to restrictive temperature ( $33^{\circ}\text{C}$ ) before the block point at the time indicated, and cells were sampled at times indicated thereafter (L1, L6, D4, L1, L6).

nificant relative labeling rate changes during the cell cycle. Among these, the fluctuations in labeling rates range from about 3- to 12-fold during the cycle. The polypeptides that demonstrate such extensive labeling rate fluctuations generally show peak labeling periods which serve to identify individual cell cycle stages.

Second, the overall cellular rate of  $^{35}\text{SO}_4^-$  or [ $^3\text{H}$ ]arginine incorporation during the cell cycle can have an overriding influence on the actual (not the relative) rate of labeling of a polypeptide. Overall  $^{35}\text{SO}_4^-$  and [ $^3\text{H}$ ]arginine incorporation rate patterns differ from each other during the cell cycle. Synchronized cells have high  $^{35}\text{SO}_4^-$  utilization rates during the light phase but incorporate [ $^3\text{H}$ ]arginine more efficiently during the dark phase. Thus, a polypeptide such as m25, which in relationship to other polypeptides is labeled by either  $^{35}\text{SO}_4^-$  or [ $^3\text{H}$ ]arginine at a greater rate in the light phase, actually incorporates [ $^3\text{H}$ ]arginine more rapidly during a pulse in the dark and  $^{35}\text{SO}_4^-$  more rapidly in the light. Circumstances such as these clearly indicate that neither  $^{35}\text{SO}_4^-$  nor [ $^3\text{H}$ ]arginine incorporation can be a direct measure of the rate of polypeptide synthesis in these cells, since incorporation rates are governed by both the specific activity of the immediate precursor pool (which relates to label uptake and precursor pool size) and the actual rate of synthesis. We have, however, measured rates of cytoplasmic and chloroplast protein synthesis during the cell cycle by means independent of precursor specific activity changes and have found that the rate changes in protein synthesis during the cell cycle are much

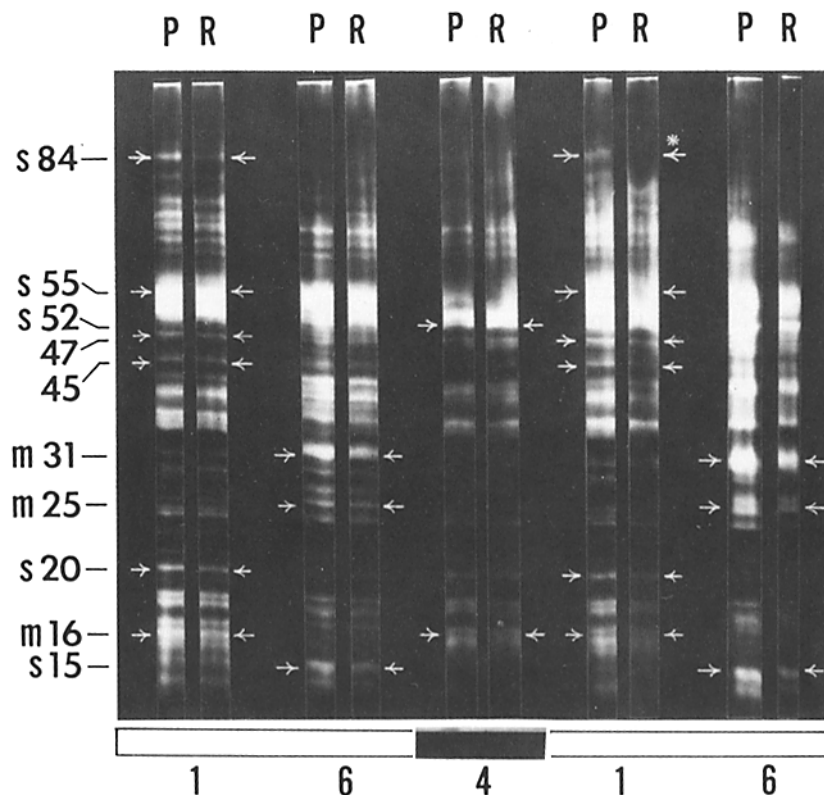


FIGURE 15  $[^3\text{H}]$ arginine labeling of polypeptides in synchronous ts10001 cultures after shift to restrictive temperature. Autoradiograms of 10% polyacrylamide gels show the pattern of polypeptides from cultures of ts10001 synchronized at permissive temperature and either retained at permissive (P) temperature or shifted to restrictive (R) temperature according to scheme in Fig. 14. Arrows point out labeled polypeptides which serve to identify cell cycle progress at both restrictive and permissive temperature. Asterisk (\*) above arrow on s84 indicates its absence in L1 at restrictive temperature. Samples analyzed by electrophoresis were taken at L1, L6, D4, L1, and L6, indicated here and in Fig. 14.

closer to the  $[^3\text{H}]$ arginine pattern than the  $^{35}\text{SO}_4$  pattern (Baumgartel and Howell, manuscript in preparation).

Third, the rate of polypeptide turnover in light-grown asynchronous cells is negligible during the cell cycle. The absence of appreciable turnover or interconversion means that the major polypeptides do not appear transiently during the cycle in light-grown cells. This seems to be the case with the exception of certain cell wall proteins which are cast off at the time of cell separation when the mother wall is shed (18). Jones et al. (15) have pointed out that there is extensive protein turnover in the dark phase in the cell cycle of synchronous cells. This was reasoned because arginine incorporation continued at a high rate in the dark in the absence of net protein accumulation. In this

study, we have not examined the turnover of protein in the dark.

The relative  $^{35}\text{S}$  or  $[^3\text{H}]$ arginine labeling pattern of specific polypeptides is shown with relationship to other cell cycle events in Fig. 16. Marker polypeptide labeling events are distributed throughout the cell cycle, but an abundance of peak polypeptide labeling events appear in the mid-light phase and in the mid-dark phase near the time of cell division. Earlier work from this laboratory showed that in these two periods (roughly the second and fourth quarters of the cell cycle), chloroplast (second quarter) and nuclear-cytoplasmic (fourth quarter) macromolecular synthetic events required for cell division are completed (12). Thus, it is not unexpected that there might be a congestion of stage-specific polypeptide labeling events



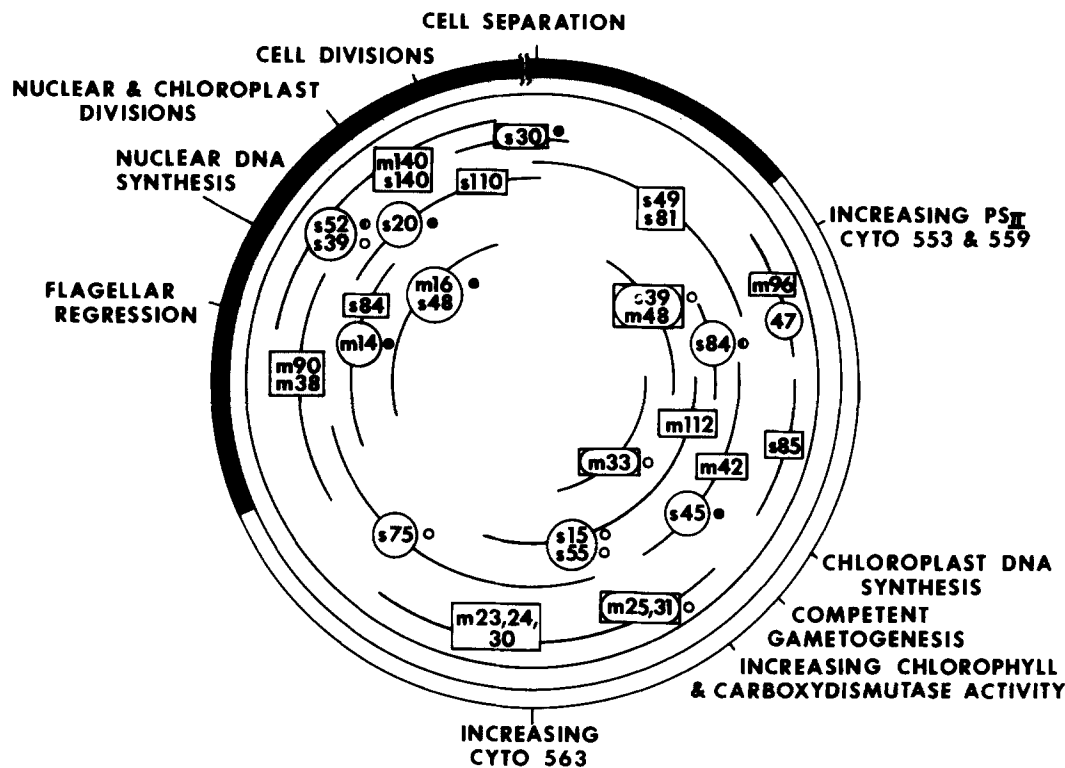


FIGURE 16 Cell cycle map of polypeptide labeling in synchronous cultures. Inner arcs show authors' interpretation of maximum relative labeling periods for selected membrane (m) or soluble (s) polypeptides. [ $^3\text{H}$ ]arginine labeling is designated by a circle, as for s75;  $^{35}\text{S}$ -labeling by a rectangle, as for s84; and labeling by both as for m33. For comparison, cell cycle events on outer circle are shown with relationship to 12-h light (light bar) and 12-h dark (dark bar) illumination cycle. A 1-h interval has been deleted from the dark-phase between cell divisions and cell separation to somewhat compensate for the difference seen between the length of this period in synchronous and asynchronous cultures. Small dot after the polypeptide designations on the inner arcs indicates whether the polypeptide is labeled in the light (○) or dark (●) in the light-grown asynchronous cells. Polypeptides that are labeled in a dark-to-light transition after 8 h in the dark (as in Fig. 10) are indicated as ◐.

at the mid-light and mid-dark phases.

A group of polypeptides that show some of the greatest changes in rate of labeling during the cell cycle are the major chloroplast membrane polypeptides (m23, m24, m25, m30, and m31) described recently by Beck and Levine (2) and designated as group II polypeptides. Hooper (9, 10) and Eytan and Ohad (8) have extensively studied the synthesis of polypeptides from this group during greening in the mutant *y-1* and have shown that the synthesis of these polypeptides is under indirect regulation by a precursor of chlorophyll. Our studies have confirmed the findings of Beck and Levine (2) that the group II polypeptides are labeled at a high relative rate in synchronous wild-type cells at a time of maximum chlorophyll accu-

mulation. Although it may seem like an apparent contradiction it is true as well, in confirmation of the findings of Bourguignon and Palade (5), that these polypeptides incorporate arginine at a greater absolute rate in the dark phase. This is because the whole cell rate of arginine incorporation is much greater when the relative rate of synthesis of these polypeptides has dropped.

An even more comprehensive analysis of the pattern of polypeptide labeling than the one described here can be obtained by using two-dimensional polyacrylamide gel electrophoresis to resolve polypeptides. Preliminary experiments in our laboratory with such gel systems demonstrate that the data base of polypeptide labeling events can be expanded by two-dimensional gels. None-

theless, our initial observations from these systems do not invalidate the results we have reported here using one-dimensional gels. It could be argued that most of the major polypeptide bands we see in one-dimensional gels are actually composite bands of many polypeptide species, and that the lack of labeling fluctuation observed for most bands results from rate-averaging in the labeling of the independent species. However, in fact, most of the major bands we see and have discussed here are composed primarily of a single species as determined by the two-dimensional gel criterion. While *C. reinhardtii* appears to have a wide complexity of polypeptides, a bulk of its protein is apparently found in the 90–100 species resolved in one-dimensional gels. Support for the notion that each major band represents primarily a single polypeptide species is discussed further in the following section in which a single condition, light, can be shown to alter the labeling of nearly every polypeptide band.

### Light-Dark Effects

Because illumination cycles are used to synchronize *C. reinhardtii* cells, an obvious concern is to distinguish between those effects that can be attributed to the cell cycle and those that simply result from the ongoing illumination conditions. We have attempted to uncover the effects related strictly to illumination conditions by carrying out light-shift experiments with asynchronous cells. When asynchronous light-grown cells are shifted to the dark, an immediate change is observed in the spectrum of [<sup>3</sup>H]arginine polypeptides. This is in contrast to dark-phase synchronous cells which do not respond extensively to immediate light-shift effects.

The pattern of polypeptide labeling during the cell cycle in light-dark synchronized cells is by and large consistent with the labeling pattern changes observed when light-grown cells are shifted to the dark. In Fig. 16 it is indicated whether stage-specific polypeptides are labeled with [<sup>3</sup>H]arginine in the light or after shift to the dark in light-grown asynchronous cells. It is clear that most polypeptides labeled in the light phase in synchronous cells are labeled in the light in asynchronous cells, and most labeled in the dark phase in synchronous cells are labeled after a shift to the dark in asynchronous cells. For example, chloroplast membrane polypeptides, m25 and m31, and the large and small subunits of RUBP-carboxylase, s15 and

s55, which are extensively labeled in the light phase in synchronous cells, are labeled in the light in asynchronous cells. In opposite fashion, m14, m16, s20, and s30, which are labeled in the dark phase in synchronous cells, are labeled when asynchronous cells are shifted to the dark.

With these observations, one might assert that the cell cycle program of polypeptide labeling in light-dark synchronized cells is a triviality based only on the pattern of polypeptides labeled in various illumination conditions. However, this simple explanation appears inadequate for several reasons. First, the pattern of polypeptide labeling in synchronous cultures does not abruptly change at the light-dark and dark-light transitions. Instead, for a given polypeptide, one generally sees a unique stage-specific pattern that gradually rises and falls. Second, the labeling pattern in synchronized cells of a couple of polypeptides, s45 and m14, does not agree with the findings in the light-shift experiments with asynchronous cells. S45, for example, is preferentially labeled during the light phase in synchronous cells, but it is labeled after shifting to the dark in asynchronous cells. Third, asynchronous light-grown cells after 8 h of dark adaptation do not have the characteristics of dark-phase synchronous cells in that the dark-adapted asynchronous cells still respond extensively to light shifts. Thus, the light-shift experiments with asynchronous cells more or less define the light requirements for the labeling of certain polypeptides, but they do not explain the dynamics of the cell cycle program.

The time of labeling of s84 during the cell cycle does make real sense with regard to its labeling behavior in light-shift experiments with asynchronous cells. This polypeptide is labeled after asynchronous cells have been dark-adapted for 8 h and then immediately shifted to the light. In the same manner in synchronous cells, s84 is labeled during the light immediately after the dark-light transition. The cell cycle labeling behavior of other polypeptides cannot be so easily explained. For example, s52 is preferentially labeled after a shift to the light in dark-adapted asynchronous cells. In synchronous cells, s52 labeling likewise increases during the dark phase, but the labeling does not require light.

The effects of illumination shifts are most obvious with regard to s15 and s55 (small and large subunits of RUBP-carboxylase). Iwanij et al. (14) have reported that arginine labeling of this chloroplast protein is highly stage-specific in synchro-

nous cells with peak labeling occurring in the light period. However, we have found in our experiments that the apparent stage-specific labeling of RUBP-carboxylase subunits is not entirely a cell cycle phenomenon but relates to the immediate illumination conditions as well. Blair and Ellis (3) have noted this light effect on the synthesis of large subunit in isolated chloroplasts, and they have attributed the effect to an energy source requirement for chloroplast protein synthesis.

### Continuing Program in a *cb* Mutant

It was the purpose of these experiments with a *cb* mutant to study the effects during the cell cycle of a *cb* mutation on the subsequent expression of a comprehensive set of cell cycle indicators. *Cb* mutants are unable to divide when shifted to restrictive temperature before their block point in the cell cycle (13). At the block point, cell division in the mutant cells becomes insensitive to temperature shifts, and the mutant cell beyond this cell cycle stage can progress through cell division at restrictive temperature. The block point, therefore, is considered to be the cell cycle stage in a *cb* mutant when the temperature-sensitive gene product required for cell division normally completes its function.

Although *cb* mutants are prevented from dividing when shifted to restrictive temperatures, it is difficult to determine how far these mutants progress beyond their block points. In several mutants, a number of cell cycle events other than cell division have been monitored when the cells are shifted to restrictive temperature. In the more interesting cases, the cell cycle clock continues for some events but stops for others. Such cases allow one to determine which cell events are conditionally related to each other (11), i.e., which events require the prior expression of an earlier event. Several *cb* mutants, including the one studied here, ts10001, continue to increase in cell envelope volume and continue to synthesize protein at restrictive temperature.

When ts10001 is shifted to restrictive temperature before its block point, it appears to continue on a cell cycle program of polypeptide labeling similar to that of the unshifted control. Instead of stopping at a single cell cycle stage and continuously producing a polypeptide pattern characteristic of that stage, the mutant appears to progress through a cell cycle program at restrictive temperature for over a cell cycle. The stage-specific label-

ing of several key polypeptides indicates that the program of labeling is largely intact (with the possible exception of s84) at restrictive temperature for over a cell generation beyond the block point.

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